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Pharmacodynamics of Pulse Dosing versus Standard Dosing: In Vitro Metronidazole Activity against *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*

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Pulse dosing is a novel approach to dosing that produces escalating antibiotic levels early in the dosing interval followed by a prolonged dose-free period. Antibiotic is frontloaded by means of four sequential bolus injections, after which antibiotic levels are allowed to diminish until the next dose. This study compares standard thrice-daily dosing and pulse dosing of metronidazole against *Bacteroides* spp. in an in vitro model. Two American Type Culture Collection *Bacteroides fragilis* isolates (metronidazole MIC for each organism = 1 mg/liter) were exposed to metronidazole for 48 or 96 h. Human pharmacokinetics were simulated for an oral 500-mg dose given every 8 h (maximum concentration of drug [C_{max}] = 12 mg/liter; half-life = 8 h; area under the curve [AUC] = 294 mg · h/liter) and for pulse dosing. Pulses, each producing an increase in metronidazole concentration of 9 mg/liter, were administered at times 0, 2, 4, and 6 h of each 24-h cycle, with a targeted half-life of 8 h (AUC = 347 mg · h/liter). A metronidazole-resistant *B. fragilis* strain (metronidazole MIC = 32 mg/liter) was exposed to both dosing regimens and, additionally, to a regimen of 1,500 mg administered once daily (C_{max} = 36 mg/liter; AUC = 364 mg · h/liter). Furthermore, regimens against one *B. fragilis* isolate and one *B. thetaiotaomicron* isolate corresponding to one-fourth and one-eighth of the thrice-daily and pulse dosing regimens, mimicking peak metronidazole concentrations achieved in abscesses, were simulated in 48-h experiments (metronidazole MIC = 1 mg/liter). Time-kill curves were generated for each experiment and analyzed for bactericidal activity, defined as a bacterial burden reduction ≥ 3 $\log_{10}$ CFU/ml. The results of paired (Wilcoxon matched-pair signed-rank test) and nonpaired (Mann-Whitney test) statistical analyses conducted on time to 3 $\log_{10}$ kill data and area under the kill curve data from each of the thrice-daily dosing experiments versus each of the pulse dosing experiments were considered not significant for a given isolate-dosing regimen combination. The thrice-daily dosing, pulse dosing, and once-daily dosing regimens all exhibited bactericidal activity. Metronidazole administered in standard or pulse dosing fashion was highly active against both susceptible and resistant strains of *Bacteroides* spp.

Since the advent of antibiotics, the infectious diseases community has struggled with optimal methods to effectively deliver these agents, including the amount needed, the interval over which the agents should be administered, and the duration of therapy. Early experimental work contrasting intermittent dosing to constant infusion showed that serum concentrations did not need to remain above the MIC for the organism for the entire dosing interval (5). Later work demonstrated that intermittent dosing and once-daily dosing, which resulted in elevated serum concentrations of antibiotic, drove tissue concentrations higher (7). Depending on the antibiotic-bacterium combination, high tissue concentrations could result in more rapid killing, and a subsequent decrease in antibiotic concentration could take advantage of a postantibiotic effect and possibly avoid adaptive resistance (2).

Pulse dosing is a novel approach to antibiotic delivery that produces escalating antibiotic levels early in the dosing interval followed by a prolonged dose-free period. This type of drug delivery technology could offer therapeutic advantages such as reduced dose frequency and greater patient compliance. In comparison to intermittent dosing, pulse dosing front loads the antibiotic, allowing an extended dose-free period during which the antibiotic concentration falls close to zero. However, unlike a single, large bolus dose given once daily (e.g., aminoglycoside single-daily dosing), short bursts of antibiotic are separated by short dose-free periods, allowing the serum concentration to fluctuate (Fig. 1). This pulse dosing technology is presently in development for several antibiotics, including metronidazole (1). Fractionating the antibiotic dose and administering the drug in a pulsatile fashion may expose the target bacteria to high concentrations of antibiotic at various phases of the bacterial growth cycle, attacking the most vulnerable bacteria in the overall population. Moreover, the bacteria are allowed a lengthy drug-free period, possibly mitigating issues of resistance.

The purpose of this investigation was to compare and contrast the activity of pulse-dosed metronidazole to standard thrice-daily dosing in an anaerobic in vitro pharmacodynamic model. The study involved sensitive and resistant *Bacteroides fragilis* isolates and one *Bacteroides thetaiotaomicron* isolate in both traditional and pulse dose simulations.

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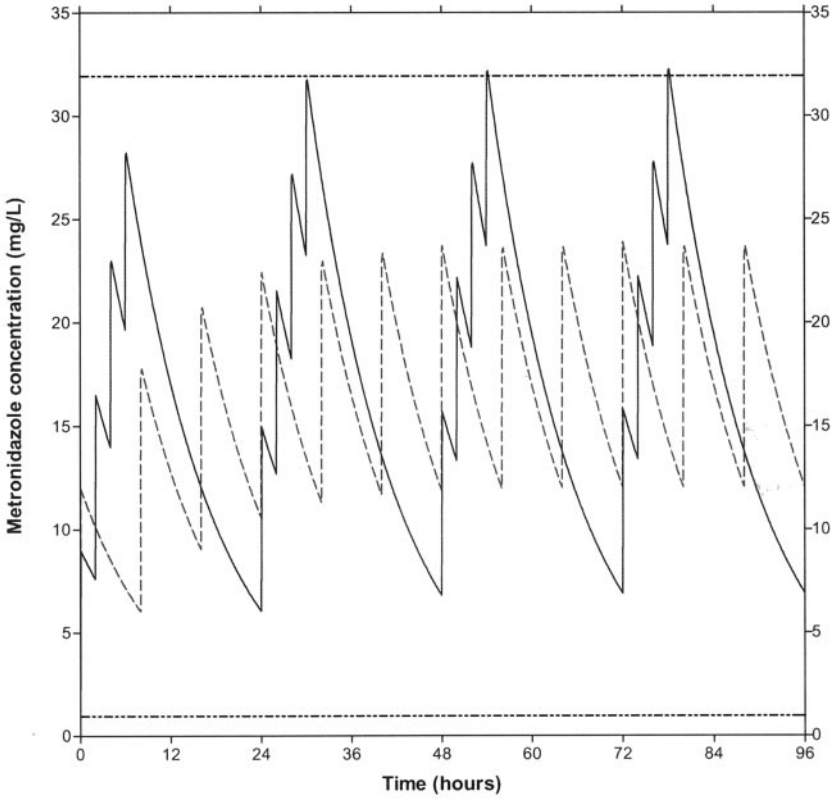

FIG. 1. Pharmacokinetic profile of standard thrice-daily dosing and pulse dosing. The solid line represents the pulse dosing profile, the dashed line represents the thrice-daily dosing, and the two dash-dot-dot lines represent the bacterial MICs of the resistant isolate (32 mg/liter) and the sensitive isolates (1 mg/liter).

MATERIALS AND METHODS

A total of 19 concentration time-kill curve experiments with metronidazole were performed in duplicate. The dosing regimens included standard thrice-daily dosing, pulse dosing, and, against the metronidazole-resistant *B. fragilis* isolate, a once-daily dosing regimen (Table 1).

In vitro model. Concentration time-kill curves were conducted using a previously described in vitro model placed in a Bactron IV anaerobic chamber (Sheldon Manufacturing, Cornelius, Oreg.) (9). Before each experiment, the isolates were inoculated into Anaerobe Broth MIC (Becton Dickinson Microbiology Systems, Sparks, Md.) and incubated anaerobically overnight for 18 to 20 h. Next, the overnight cultures were diluted 1:10 in fresh medium, reincubated for 1 h, and compared to a 0.5 McFarland turbidity standard to estimate the amount of inoculum to inject into the model to achieve a starting inoculum of approximately 10^6 CFU/ml. Growth control experiments were conducted in duplicate for each of the isolates. Antibiotic administration was achieved by a bolus metronidazole injection at time 0 h. The thrice-daily dosing experiments involved additional metronidazole boluses at times 8 and 16 h of each 24-h cycle, while the pulse-dose experiments involved additional boluses at times 2, 4, and 6 h of each 24-h cycle. Each experiment was run in duplicate for 96 (6 experiments) or 48 (13 experiments) h. Antibiotic-free medium was pumped via a peristaltic pump into

TABLE 1. Time-kill experiments

Organism	Metronidazole MIC (mg/liter)	Dose interval (h)[a]	Dose fraction	Change in concn per bolus (mg/liter)	AUC (mg · h/liter)
B. fragilis ATCC 25285	1	8	1	12	294
		8	1/4	3	73.5
		8	1/8	1.5	36.8
		Pulse	1	9	347
		Pulse	1/4	2.25	86.8
		Pulse	1/8	1.125	43.4
B. fragilis ATCC 23745	1	8	1	12	294
		Pulse	1	9	347
B. fragilis EL9906	32	24	1	36	364
		8	1	12	294
		Pulse	1	9	347
B. thetaiotaomicron ATCC 29741	1	8	1/4	3	73.5
		8	1/8	1.5	36.8
		Pulse	1/4	2.25	86.8
		Pulse	1/8	1.125	43.4

[a] Growth control experiments were conducted in duplicate for each organism. Pulse interval constitutes four pulses given at 0, 2, 4, and 6 h of every 24-h period.

TABLE 2. Time to 3-log kill

Organism	Dose fraction	Time to 3-log kill[a]	
		Pulse	Thrice daily
B. fragilis ATCC 25285	1	6.16	8.24
B. fragilis ATCC 25285	1/4	7.28	6.08
B. fragilis ATCC 25285	1/8	8.64	8.96
B. thetaiotaomicron ATCC 29741	1/4	1.68	3.12
B. thetaiotaomicron ATCC 29741	1/8	3.84	21.52
B. fragilis EL9906	1	10.56	25.04
B. fragilis ATCC 23745	1	4.60	5.76

[a] $P > 0.05$ for all experiments.

the closed system to displace an equal volume of antibiotic-containing medium, simulating a monoexponential pharmacokinetic process and producing the desired half-life ($t_{1/2}$) of 8 h.

Bacteria and susceptibility testing. Two American Type Culture Collection isolates of *B. fragilis* (ATCC BF 25285 and BF 23745; metronidazole MIC = 1 mg/liter for each organism) and one *B. thetaiotaomicron* isolate (BT 29741; metronidazole MIC = 1 mg/liter) were used in the experiments. Additionally, one resistant *B. fragilis* isolate (EL 9906; metronidazole MIC = 32 mg/liter), kindly provided by S.L. Pendland (University of Illinois at Chicago, Chicago, Ill.), was used. MICs for each isolate pre- and post-antibiotic exposure were determined by broth microdilution following NCCLS guidelines. *B. fragilis* ATCC 25285 was used as a quality control strain. Further MIC testing was performed with isolate EL 9906 at inocula of 4×10^5 and 5×10^7 CFU/ml to examine the effect of higher inocula. The metronidazole concentrations in these trays ranged from 20 to 80 mg/liter, increasing in increments of 4 mg/liter between 20 and 40 mg/liter and in increments of 8 mg/liter between 40 and 80 mg/liter. This allowed us to more closely determine the actual MIC.

Antibiotics. A stock solution of metronidazole (SCS Pharmaceuticals, Chicago, Ill.) was prepared by reconstitution with the appropriate amount of sterile distilled water to yield a final concentration of 100 mg/ml and was either stored at 4°C or frozen at −80°C until needed for individual experiments. Individual boluses were administered manually at the appropriate time points to produce the targeted concentration increases shown in Table 1.

Pharmacokinetics. Four groups of experiments were performed: (i) standard thrice-daily dosing versus pulse dosing, (ii) once-daily dosing versus pulse dosing, (iii) one-fourth standard dosing versus one-fourth pulse dosing, and (iv) one-eighth standard dosing versus one-eighth pulse dosing. In the first group of experiments simulating standard thrice-daily dosing, a sufficient amount of the stock metronidazole solution was injected into the model to achieve initial peak concentrations equal to free concentrations reported in humans following the administration of 500 mg of metronidazole orally (12 mg/liter). Dosing was repeated every 8 h. The simulated area under the curve (AUC) was 294 mg · h/liter. Pulse doses in the first group of experiments consisted of metronidazole boluses injected at times 0, 2, 4, and 6 h every 24 h, producing a change in concentration of 9 mg/liter per bolus and a comparable AUC to that of traditional thrice-daily dosing (pulse AUC = 347 mg · h/liter). Initially, this group of experiments was performed over 96 h. However, no change was noted in the bacterial burden between 48 and 96 h (after six experiments), so the remaining experiments in this group (three experiments), as well as those in the other three groups, were conducted over 48 h. The once-daily dosing experiment bolused a single dose of metronidazole at time 0 h to achieve a maximum concentration of drug (C_{max}) of 36 mg/liter. The pulse dose experiment conducted in this group was the same as in the first group (four boluses; 9 mg/liter each). The third and fourth groups each included thrice-daily dosing (s) and pulsatile dosing (p), simulating concentrations equal to one-fourth ($bolus_s$ = 3 mg/liter; $bolus_p$ = 2.25 mg/liter) and one-eighth ($bolus_s$ = 1.5 mg/liter; $bolus_p$ = 1.125 mg/liter) of the above-mentioned concentrations for standard dosing and pulse dosing, respectively. These fractional doses more accurately represent concentrations of metronidazole that may be achieved in abscesses (4). Pharmacokinetic schematics of the standard thrice-daily dosing and the pulse dosing are shown in Fig. 1.

HPLC. The concentrations of metronidazole were determined from batched stored samples (frozen at −80°C) in Anaerobe Broth MIC using a previously described method of high performance liquid chromatography (HPLC) with slight modifications (Scientific Research Consortium, Inc., St. Paul, Minn.) (3). The HPLC assay was linear over a range of 0.2 to 25 mg/liter, with an $r^2 \geq 0.9998$. Intraday and interday coefficients of variation were ≤1.29 and ≤0.87%, respectively.

Pharmacodynamics. At predetermined timed intervals, 1-ml samples of broth were removed from the model for bacterial quantification via serial saline dilution. Antibiotic carryover was minimized by the sequential dilution. The number of predetermined timed intervals for sampling was dependent on the frequency of antibiotic administration; however, a minimum of 21 samples were removed in the 96-h experiments, and a minimum of 11 samples were removed in the 48-h experiments. Bacterial counts were performed following a series of 1:10 dilutions of 100 μl of sample into saline and plating onto CDC Anaerobic Blood Agar plates (Becton Dickinson Microbiology Systems, Cockeysville, Md.). After anaerobic incubation for 48 to 96 h at 37°C, colony counts were performed visually. The theoretical lower limit of bacterial counting accuracy was 300 CFU/ml. Concentration time-kill curves were constructed by plotting the $\log_{10}$ CFU per milliliter values versus time.

Time to 3 $\log_{10}$ kill (T3K), determined visually using computer coordinates generated with GraphPad Prism 3.02 (GraphPad Software Inc., San Diego, Calif.), was defined as the time required for the initial bacterial burden to be reduced by 3 $\log_{10}$ CFU/ml. Bactericidal activity was defined as a drop in the starting bacterial inoculum by at least 3 $\log_{10}$ CFU/ml (99.9% killing). Area under the kill curve was calculated using GraphPad Prism 3.02 and the trapezoidal rule on the basis of actual datum points (not using the connecting lines). Due to the fact that (depending on the dosing regimen) different numbers of samples were taken between the different experiments, AUC calculations were limited by available data. To account for slight differences in starting inocula, an area value restricted by a baseline equal to the starting inoculum minus 3 $\log_{10}$ CFU/ml was calculated (AUKC3LK).

Statistics. T3K and AUKC3LK data were evaluated using GraphPad InStat 3.0 software (GraphPad Software Inc.). Paired (Wilcoxon matched-pair signed-rank test) and nonpaired (Mann-Whitney test) statistical analyses using the data in Table 2 and Table 3 were performed for each isolate-dosing regimen combination. Significance was defined as $P \leq 0.05$.

RESULTS

MICs. All MICs conducted post-antibiotic exposure fell within one tube dilution of pre-antibiotic exposure values (data not shown). MIC testing performed with isolate EL 9906 at a higher inoculum (5×10^7 CFU/ml) showed no apparent inoculum effect.

Pharmacokinetics. Comparing metronidazole concentrations attained in the model (determined by HPLC) to the expected concentrations verified the simulated pharmacokinetic parameters (half-life and peak concentration). Actual concentrations ranged from 90.8 to 127.5% of the expected concentrations, with a mean of 101.2% and a standard deviation of 7.1%. Half-lives calculated using the actual concentrations ranged from 6.9 to 9.2 h.

Time-kill kinetics. In the thrice-daily dosing experiments, the pulse dosing experiments, and the once-daily experiment conducted with the sensitive and resistant isolates at both full doses and fractional doses, colony counts were reduced by at

TABLE 3. Area under the kill curve limited by a baseline equal to starting inoculum minus 3 $\log_{10}$ CFU/ml ($AUKC_{3LK}$)

Organism	Dose fraction	$AUKC_{3LK}$[a]	
		Pulse	Thrice daily
B. fragilis ATCC 25285	1	13.83	10.92
B. fragilis ATCC 25285	1/4	6.42	5.67
B. fragilis ATCC 25285	1/8	10.65	6.31
B. thetaiotaomicron ATCC 29741[b]	1/4	NA	NA
B. thetaiotaomicron ATCC 29741	1/8	8.25	54.36
B. fragilis EL9906	1	19.32	29.68
B. fragilis ATCC 23745	1	11.80	11.59

[a] $P > 0.05$ for all experiments. NA, not applicable.

[b] $AUKC_{3LK}$ data are not available for ATCC 29741 secondary to rapid rate of kill (not enough data points above the 3-$\log_{10}$ kill baseline).

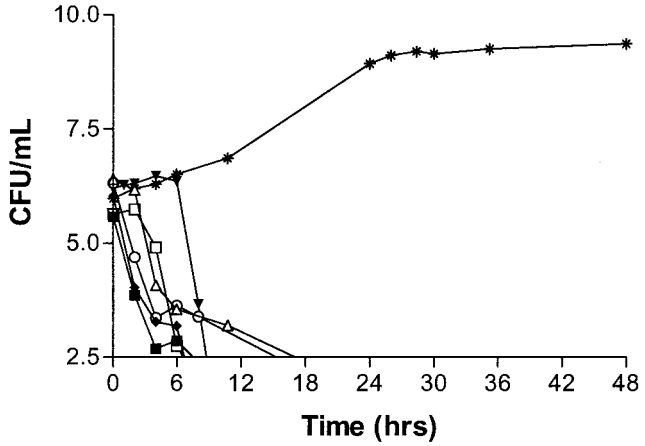

FIG. 2. Time-kill curve of *B. fragilis* BF 25285 with metronidazole pulse full dose (open square), one-fourth pulse dose (solid square), one-eighth pulse dose (open triangle facing up), standard thrice-daily dose (solid triangle facing down), one-fourth thrice-daily dose (solid diamond), and one-eighth thrice-daily dose (open circle). Growth control is depicted with asterisks.

least 3 $\log_{10}$ CFU/ml (Fig. 2 and 3 show data for the sensitive *B. fragilis* isolate BF 25285 and for the resistant *B. fragilis* isolate EL9906). Therefore, all metronidazole regimens achieved bactericidal killing. The rates of killing were similar for a given isolate-dosing regimen combination, and there were no statistically significant differences between the various regimens. The results of comparisons of T3K for each isolate-dosing regimen combination were considered not significant (Table 2). The results of analyses of AUKC3LK between experiments were also considered not significant (Table 3). Use of the resistant *B. fragilis* isolate and the thrice-daily dosing, the pulse dosing, and the once-daily dosing regimens resulted in regrowth at 48 h (Fig. 3). In contrast, in the experiments conducted with the sensitive strains (both *B. fragilis* and *B. thetaiotaomicron*) at full and fractional doses, regrowth occurred in only one of the duplicate models against one of the two sensitive *B. fragilis* isolates exposed to the thrice-daily dosing regimen. Regrowth did not occur in the *B. thetaiotaomicron* experiments or in any of the pulse dosing experiments conducted with the sensitive isolates. The organism that regrew in the single model was confirmed to be *B. fragilis*, but there was no increase in the metronidazole MIC.

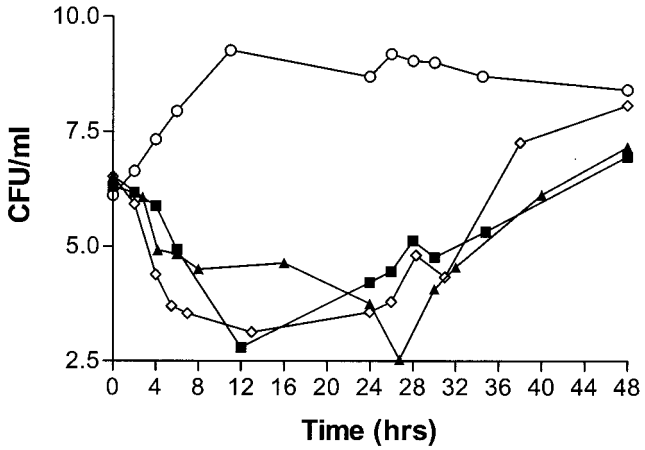

FIG. 3. Time-kill curve of resistant *B. fragilis* isolate EL 9906 with metronidazole pulse full dose (solid square), standard thrice-daily dose (solid triangle facing up), and once-daily dosing (open diamond). Growth control is depicted with open circles.

DISCUSSION

While the efficient killing of the sensitive *B. fragilis* and *B. thetaiotaomicron* strains by metronidazole was not surprising, the killing observed with the thrice-daily dosing, the pulse dosing, and the once-daily dosing regimens against the resistant *B. fragilis* isolate was unanticipated. The thrice-daily dosing metronidazole concentrations never exceeded the MIC of the resistant isolate, while the pulse dosing concentrations briefly reached the MIC after the fourth pulse on day 2. The once-daily dosing concentrations briefly exceeded the MIC after each bolus. As with previous reports of *Helicobacter pylori* experimental results, the killing rate was slower and the killing extent was less for all three dosing regimens against the resistant strain compared to the sensitive strain (6). However, we observed a discordance in our dynamic in vitro model between MICs and metronidazole activity, a finding which is similar to those of other investigators (10). To explain this killing phenomenon, we postulate some involvement of the dynamic environment created in the in vitro pharmacodynamic model. Metronidazole requires intracellular reduction to exert its activity in bacteria, and drug uptake into the cell occurs via diffusion (6, 8). Intracellular metabolism, which drives the diffusion concentrations, may be subdued in a static environment (e.g., MIC testing) (11). However, unlike the static MIC testing environment, the in vitro model environment is continually supplemented with nutrients and exposed to fluctuating antibiotic levels. Potentially, due to the dynamic nature of the in vitro model that emulates the in vivo situation, metronidazole metabolism may be increased, driving the concentration gradient and allowing more drug to be reduced to the active form.

Treatments with metronidazole dosed in the traditional thrice-daily fashion, in the once-daily fashion, or in the novel pulse dosing fashion all demonstrated activity against the *Bacteroides* spp. tested. For a given isolate, differences between the dosing methods were not significant and activity extended to the resistant isolate. This was also true for the *B. fragilis* and *B. thetaiotaomicron* isolates tested at one-fourth and one-eighth dose fractions. While this study does not demonstrate a superior response to dosing metronidazole in a pulsatile fashion against *B. fragilis* and *B. thetaiotaomicron* isolates, the effect is comparable to that of conventional dosing regimens. Perhaps this novel dosing strategy would prove advantageous against other pathogens.

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